

Daytime variation in hepatitis C virus replication kinetics following liver transplant

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RESEARCH ARTICLE

REVIS **Daytime variation in hepatitis C virus replication kinetics following liver transplant [version 2; referees: 5 approved]**

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Abstract

Background: There is a growing interest in the role of circadian regulated pathways in disease pathogenesis.

Methods: In a cohort of hepatitis C virus (HCV) infected patients undergoing liver transplantation, we observed differences in early viral infection kinetics of the allograft that associated with the time of liver transplant.

Results: A higher frequency of subjects transplanted in the morning showed a rebound in viral RNA levels (n=4/6) during the first week post-surgery. In contrast, no viral rebound was observed in seven subjects transplanted in the afternoon. None of the other parameters previously reported to influence viral replication in the post-transplant setting, such as donor age, cold-ischemia time and length of surgery associated with viral rebound.

Conclusions: These observation highlights a role for circadian processes to regulate HCV infection of the liver and warrants further investigation.

Keywords

HCV, Circadian rhythm, liver transplant, allograft, viral rebound, time of day

Open Peer Review

Referee Status:

	Invited Referees				
	1	2	3	4	5
version 2 published 20 Sep 2018					
			report		
version 1 published 07 Aug 2018			?		
	report	report	report	report	report

- William L. Irving** , University of Nottingham, UK
- Salim I. Khakhoo**, University of Southampton, UK
- Koichi Watashi**, National Institute of Infectious Diseases, Japan
- Graham R. Foster**, Queen Mary University of London, UK
- Arvind H. Patel** , University of Glasgow, UK

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Author roles: **Zhuang X:** Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Lai AG:** Formal Analysis, Methodology, Resources, Software, Writing – Original Draft Preparation, Writing – Review & Editing; **McKeating JA:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Rowe I:** Conceptualization, Formal Analysis, Investigation, Project Administration, Resources, Validation, Writing – Original Draft Preparation; **Balfe P:** Conceptualization, Investigation, Methodology, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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First published: 07 Aug 2018, 3:96 (doi: [10.12688/wellcomeopenres.14696.1](https://doi.org/10.12688/wellcomeopenres.14696.1))

REVISED Amendments from Version 1

In this revision we have responded to 3 points raised by the referees by :

- 1) Replacing reference 11 with a newer estimate of HCV incidence rates and updating the number in the text.
- 2) Rewording the text describing Figure 3B to use the word "suggesting" rather than "supporting"
- 3) Inserting into the legend for Figure 3 the numbers of patients in each graph.

See referee reports

Introduction

The circadian clock is an evolutionarily conserved biological time-keeping system that synchronizes behavioural and physiological processes to a 24-hour cycle, including cell proliferation and metabolism¹. The circadian system is recognized to regulate host innate and adaptive immune responses to microbial pathogens to conserve energy utilization^{2–8}. The circadian system comprises a central clock in the suprachiasmatic nucleus of the hypothalamus and secondary clocks in the peripheral organs. The liver is a highly circadian regulated organ with up to 20% of genes under clock control⁹. Research over the past two decades has demonstrated that disrupting clock function associates with the development of liver diseases, including fatty liver disease, cirrhosis and hepatocellular carcinoma (HCC), highlighting a key role for the circadian system in regulating hepatic function^{9,10}.

Viral infection of the liver is a global health problem with up to 71 million individuals infected with hepatitis C virus (HCV) that causes progressive liver disease and is one of the leading indications for liver transplantation¹¹. In almost every case HCV infects the newly transplanted organ or donor allograft, providing an unprecedented window to study the early stages of HCV infection. We had the opportunity to study the relationship between the time of liver transplantation and HCV replication dynamics in subjects enrolled in a clinical trial to assess the safety and efficacy of an entry inhibitor targeting scavenger receptor BI (SR-BI)¹². We noted differences in viral infection of the allograft in control subjects that associated with the time of liver transplant, suggesting a role for circadian processes to regulate HCV entry into the liver.

Results

HCV infection of the newly transplanted graft is reported to show "rapid" or "slow" early phase replication kinetics (Figure 1)^{13,14}, however, the host pathways defining these profiles are not well understood. To investigate whether HCV replication kinetics is influenced by the time of transplant, patients in the untreated arm of the trial were grouped according to their time of surgery between the hours 6am–1pm (AM) (n=6) or 2pm–11pm (PM) (n=7). No patients were transplanted during the night (11pm–6am). Transplantation was required for liver failure (n=8) or HCC (n=5). Patients were infected with HCV genotype (Gt) 1 (7 patients) or Gt3 (4 patients), with single cases of Gt2 and

Gt4. No significant differences in baseline median HCV RNA load were observed ($5.4 \log_{10}$ IU/ml) (Table 1)¹⁵. Additional clinical parameters previously reported to affect HCV replication or allograft survival, such as donor age (AM: median, 55 years; range 45–69 years; PM: median, 44 years; range, 29–64)¹⁶, cold-ischemia time¹⁷, duration and time of operation¹⁸ were comparable in the AM or PM groups (Table 1). In four of six patients (#3, 7, 8 and 11) in the AM group, a viral rebound toward pre-transplant levels was observed during the time of study (Figure 2A). In contrast, none of the seven patients transplanted in the PM group showed a recovery of viral load to pre-transplant levels (Figure 2B).

Combining the replication kinetics from control subjects within AM or PM groups enabled us to apply lines of best fit and to conclude that the differences in replication kinetics were significant (Figure 3A) (F-test, $p < 0.001$). A similar analysis of patients receiving the SR-BI antagonist ITX5061 failed to show any significant difference in replication kinetics between the AM (n=4) or PM (n=6) groups (Figure 3B), suggesting a role for circadian regulation of HCV entry.

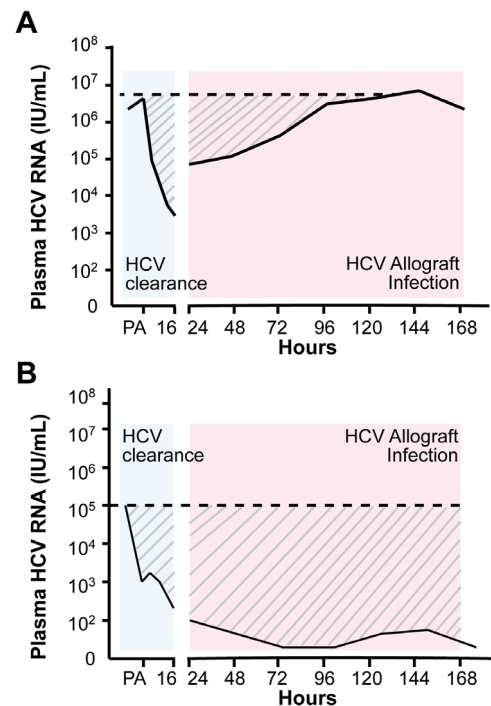


Figure 1. Schematic of hepatitis C virus (HCV) replication kinetics in the first week following liver transplant. Representative plasma HCV RNA levels are shown over the first week post-transplant. The declining values observed in the first 24 h following the post-anhepatic (PA) phase represent viral clearance (blue shading) from the periphery. Infection of the allograft results in a subsequent increase in viral RNA that can occur with either 'rapid' (A) or 'slow' (B) kinetics (pink shading). The horizontal dashed line represents the plasma viral load pre-transplant and allows us to quantify viral replication kinetics by measuring the area under the curve (AUC) (the hatched area), a patient showing rapid infection will result in a smaller AUC value compared to one with slower kinetics.

Table 1. Cohort data. Values shown for continuous variables are means (standard deviations).

Variable	AM (n=6)	PM (n=7)
Age, years	53 (11)	54 (7)
Sex		
Male	5	6
Female	1	1
Ethnicity		
Caucasian	4	5
Weight, kg	85 (11)	82 (16)
Indication for transplant		
Liver failure	3	5
HCC	3	2
MELD score	13 (4.5)	15 (3.7)
Initial HCV RNA, log ₁₀ IU/ml	5.0 (1.5)	5.7 (0.6)
Genotype		
Gt1	2	5
Non-Gt1	4 (2 Gt3, 1 Gt2, 1 Gt4)	2 (2 Gt 3)
Donor data		
Age, years	56 (8)	45 (11)
Cold Ischaemic time, mins	534 (64)	583 (142)
Duration of operation, h†	5.7 (2.0)	4.5 (0.8)

†Duration estimated between the start of anhepatic phase and arrival on intensive care unit. One patient's operation (AM) was 10 hours, all others were 4–6 hours. HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; HCV, hepatitis C virus.

We¹² and others^{13,14} have previously reported a rapid decrease in HCV RNA within the first 16 hours following surgery due to clearance of virus in the periphery by the reticular endothelial system of the new liver. To assess the influence of transplant time on viral clearance and allograft infection kinetics we calculated the area over the infection curve for control and treated subjects between 0–16 h and 24–168 h after transplantation (Figure 3C, D). Time of transplant had minimal effect on viral clearance in all subjects (Figure 3C, D). Control patients in the AM group showed higher rates of infection than those in the PM group, whereas this pattern was not apparent in the ITX5061-treated groups (AM, n=6; PM, n=4) and response to therapy was not time-dependent (Figure 3C, D). Structuring the data in this manner did not reach significance and this most likely reflects the small number of data points analysed relative to the earlier regression analysis (Figure 3A, B). In summary, these data support an association between HCV allograft infection rate and time of liver transplantation.

Discussion

Our observation that the time of day of liver transplantation is associated with HCV allograft infection kinetics supports a role for circadian components to regulate host pathways important

for HCV entry and replication. This observation has biological plausibility, since factors known to control both HCV entry and replication are reported to be under circadian control. For example, the tight junction proteins occludin and claudin-1, which define HCV entry into hepatocytes, have been reported to be circadian regulated in the colon¹⁹, supporting a model where HCV entry into the liver may be circadian regulated. Similarly, the abundant liver-specific microRNA, miR-122, an essential host factor required for HCV RNA replication, is circadian regulated, and miR122 targeted genes showed clear circadian profiles²⁰, providing a further pathway for circadian control of HCV RNA replication.

Natural killer cells²¹ and interferons²² are major contributors to anti-viral responses, and are reported to be circadian regulated. In the context of liver transplantation, where recipients are immunosuppressed, the impact of recipient or allograft innate and adaptive immunity may be compromised, suggesting that differences in viral kinetics may reflect differences in hepatocellular permissivity to support HCV infection.

We recognize the limitations of this analysis, particularly with respect to the small number of patients studied. There are obvious ethical constraints in accessing donor liver tissue to

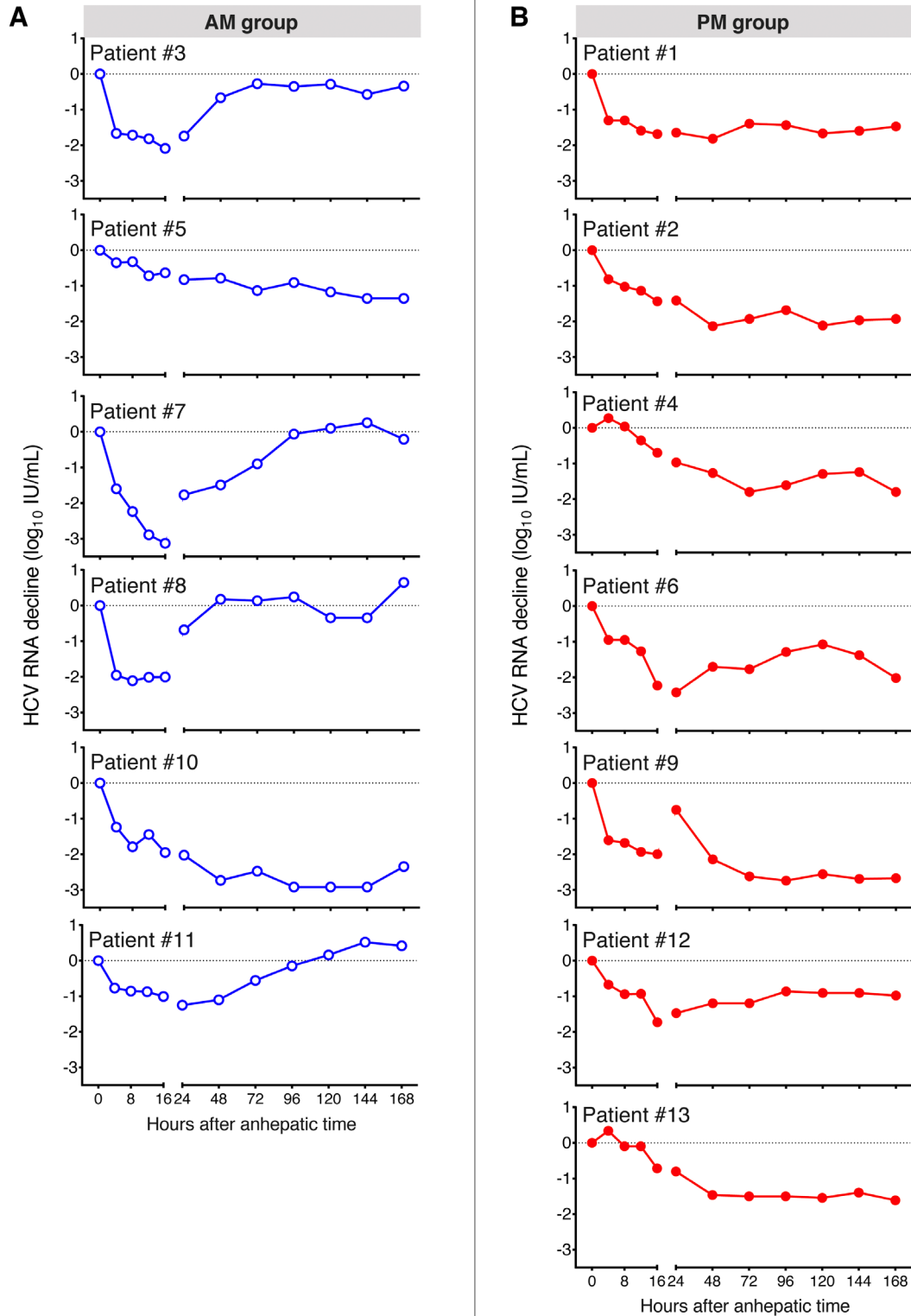


Figure 2. Hepatitis C virus (HCV) replication kinetics in control subjects in the AM or PM transplant groups. HCV RNA levels were quantified in subjects undergoing liver transplant in the AM (A) or PM (B) groups, with data expressed relative to the mean value of three samples collected after admission to hospital and before surgery. Samples were collected at 0, 4, 8, 12, 16 and 24 h post-transplant and daily thereafter for 7 days (168 h).

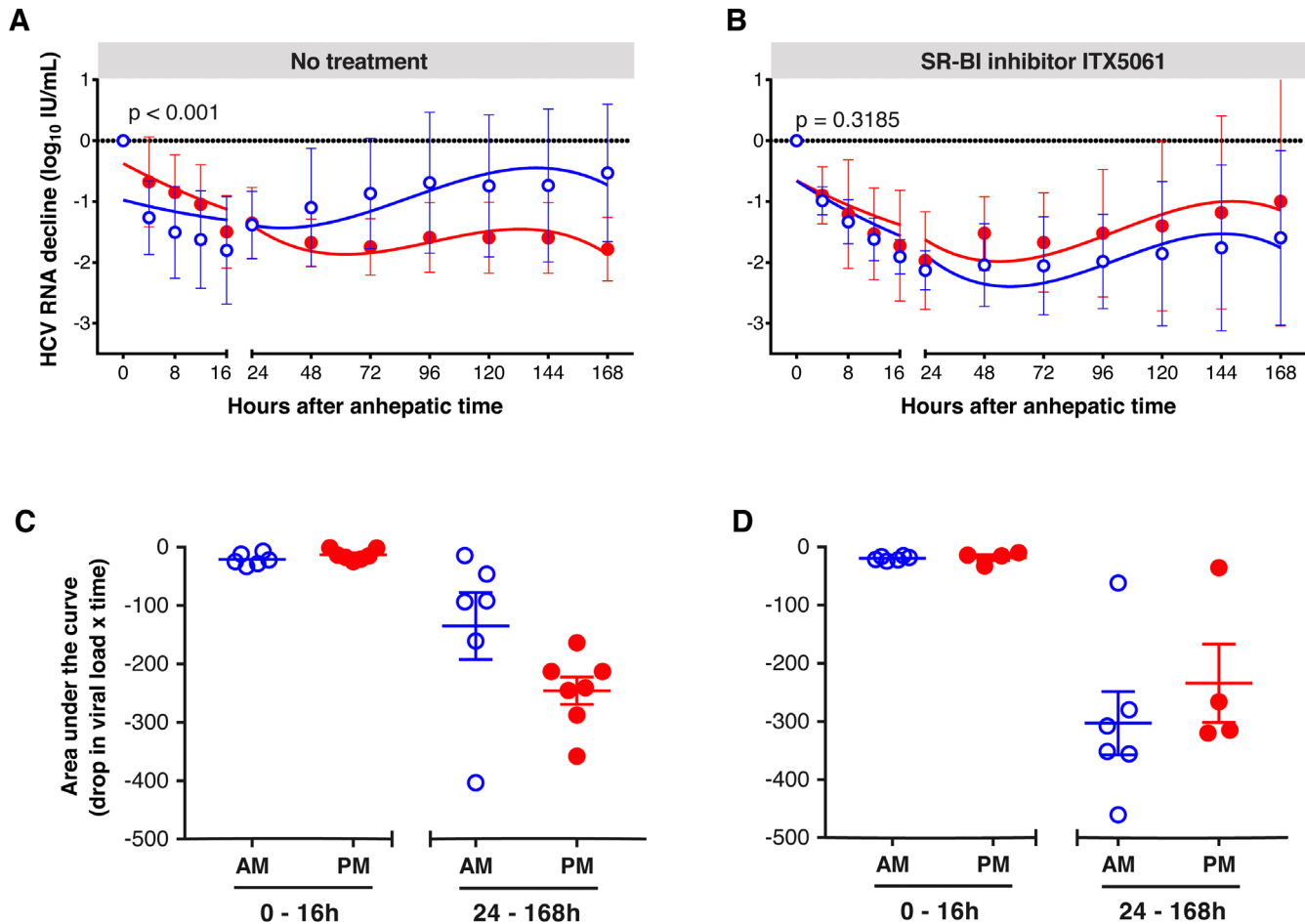


Figure 3. Effect of liver transplantation time on hepatitis C virus (HCV) clearance and early replication kinetics in control (n=13) and ITX5061 (n=10) treated subjects. The decline in HCV RNA levels in control (A) and ITX5061 treated (B) subjects in AM (blue) and PM (red) groups were averaged at each time point, plotted and lines of best fit calculated. Statistical comparison (F-test) showed a significant difference between viral replication in the control AM and PM groups ($p < 0.001$). Patients were assessed for viral clearance (0–16 h) and infection kinetics (24–168 h) by determining the AUC over time in control (C) and ITX5061 treated (D) subjects where each symbol represents a patient. Groups were compared using a student's unpaired t-test and no significant difference was observed.

assess its circadian status. However, to the best of our knowledge this is the first report highlighting a potential role for liver time-of-day regulated pathways to modulate HCV replication *in vivo* and this has clear translational potential for other hepatotropic infectious agents and the design of therapeutics.

Methods

Subjects

The data presented were obtained from subjects enrolled in an open-label phase 1b study to assess the effect of ITX5061 in patients undergoing liver transplantation at a single centre (Queen Elizabeth Hospital Birmingham, Birmingham, UK), described previously¹². All patients gave informed written consent and ethical approval was given by the UK National Research Ethics Service (reference 10/H0301/36)¹². The study was registered at clinicaltrials.gov (NCT01292824). The study enrolled men and women between the ages of 18 and 65 years who were suitable for liver transplantation. Patients with HCV-associated

end-stage liver disease or HCC were enrolled regardless of their infecting Gt or previous anti-viral treatment. Patients co-infected with HBV or human immunodeficiency virus were excluded, as were patients receiving a liver from a HCV-positive donor.

Plasma collection and analysis

Plasma was collected at screening, before surgery, at the time of transplantation, and during a follow-up period of 90 days. HCV RNA levels were measured on admission to the hospital, immediately following the induction of anesthesia, at the time of portal vein clamping (the start of the anhepatic phase), immediately before perfusion of the allograft, and 1 hour later. Plasma samples were collected every 4 hours during the first post-transplant day, daily for the first week, weekly for the first month, and monthly thereafter up to 90 days. Plasma HCV RNA was measured using the COBAS TaqMan HCV test version 2.0 (Roche Diagnostics Ltd., Switzerland) in a laboratory accredited

by the Health Protection Agency UK. Data were analysed using t-tests or F-tests in GraphPad Prism 7.0 software.

Data availability

Raw data for the study, including demographic information for untreated transplant patients and hepatitis C virus RNA levels in untreated and ITX5061-treated groups, are available on OSF: <http://dx.doi.org/10.17605/osf.io/kjnh15>. Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Grant information

This work was supported by Wellcome Trust (through Institutional Strategic Support Funds awards and grant No. 200838 to J.A.M.), the PERCAT Research Development Fund at the University of Birmingham and by European Union’s Horizon 2020 research and innovation programme (grant agreement No. 667273).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Referee Status:



Version 2

Referee Report 28 September 2018

doi:[10.21956/wellcomeopenres.16122.r33952](https://doi.org/10.21956/wellcomeopenres.16122.r33952)



Koichi Watashi

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

The authors appropriately responded to the reviewer's comments with opening the current available information.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 21 August 2018

doi:[10.21956/wellcomeopenres.16003.r33655](https://doi.org/10.21956/wellcomeopenres.16003.r33655)



Arvind H. Patel 

MRC (Medical Research Council) - Centre for Virus Research, University of Glasgow, Glasgow, UK

This is an interesting study investigating the role of the circadian clock in hepatitis C virus (HCV) infection and replication. Using samples of HCV-infected liver transplant recipients enrolled in a clinical trial assessing the efficacy of a virus entry inhibitor ITX5061, the authors investigated replication kinetics of the virus and asked whether they are affected by the timing of the allograft. Previous studies have reported rapid or slow early phase virus replication in the new graft but the mechanisms remain unknown. Here the authors determined viral load at multiple time points up to 1 week in samples collected from untreated individuals who received the new liver in the morning (n=6, the AM group) or in the afternoon (n=7, PM). Remarkably, in 4 out of 6 patients in the AM group viral rebound approaching pre-transplant levels was observed. In contrast, no increase in viral load was seen in any of the patients in the PM group. This difference was statistically significant allowing the authors to suggest that circadian processes play a role in regulating HCV infection. The authors also performed similar analysis in transplant recipients who were treated with the virus entry inhibitor ITX5061 and found no significant difference in the viral replication kinetics between the AM and PM groups allowing the authors to suggest circadian regulation of HCV entry. Notwithstanding the small sample size, the authors' observations are very interesting and warrant

further investigation to elucidate pathways and regulation of viral and host factors that modulate viral infection and replication in vivo.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 21 August 2018

doi:[10.21956/wellcomeopenres.16003.r33652](https://doi.org/10.21956/wellcomeopenres.16003.r33652)



Graham R. Foster

The Liver Unit, Blizard Institute, Queen Mary University of London, London, UK

This is an interesting short report examining the hypothesis that innate factors facilitating the early stages of the HCV replication cycle are under circadian control. The authors suggest that HCV entry and early replication is reduced in the afternoon. In support of this they show data from a detailed analysis of viral kinetics post liver transplantation and they compare a group undergoing surgery in the morning with those transplanted in the afternoon. The groups are reasonably well matched and obvious co-founders (genotype, duration of surgery) are accounted for. I presume that all donors were heart beating and that there were no gross differences between them but it would be helpful if the authors could confirm this and exclude the possibility that the observations are due to the use of 'poor quality' organs from non-heart beating donors in the operations performed in the afternoon. It would also be helpful to add in the viral genotypes to Figure 2 – there are some differences in the patterns within the groups and it would be helpful to see the figures annotated with viral genotype.

The authors are appropriately guarded in their conclusions and make sensible suggestions regarding the underlying mechanisms. However, I would be interested in their thoughts on whether this finding should promote consideration of studies varying the timing of the administration of all oral antiviral agents – I wonder if drugs administered in the afternoon when viral spread is less active might be more effective and

permit reduced treatment durations compared to medication taken in the morning.

Minor comments:

The reference to the total population infected with HCV is a little old and more recent estimates suggest that the prevalence is somewhat less than reported here.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: I have received speaker and consultancy fees from companies marketing drugs for the treatment of hepatitis C - specifically AbbVie, Gilead and MSD

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 04 Sep 2018

Peter Balfe, University of Birmingham, UK

Referee : "it would be helpful to see the figures annotated with viral genotype."

Response : Please see on line data where this data is given

Referee : "I wonder if drugs administered in the afternoon when viral spread is less active might be more effective and permit reduced treatment durations compared to medication taken in the morning."

Response : An interesting idea that could be relevant to anti-viral treatment for a range of human and animal pathogens. Our observations may inform future the design of pre-clinical studies assessing PK and drug efficacy.

Referee : "The reference to the total population infected with HCV is a little old and more recent estimates suggest that the prevalence is somewhat less than reported here.

Response : We've updated this citation to the more recent WHO report from 2017, which suggest 71 million chronic infections exist (

<http://www.who.int/entity/hepatitis/publications/global-hepatitis-report2017/en/index.html>)

Competing Interests: No competing interests were disclosed.

Referee Report 21 August 2018

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Koichi Watashi

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Zhuang and colleagues reported the difference in HCV recurrent dynamics that was concluded to depend on the time of transplantation in a small scale number of patients after liver transplantation: They showed that patients transplanted in the morning showed a high frequency of rapid HCV recurrence compared with those transplanted in the afternoon. No patients receiving transplantation in the afternoon showed a HCV rebound to the original level before transplantation.

This paper shows a clinical evidence prospecting a surprising idea that HCV recurrence is reflected by circadian. Although it would be better to have more patient numbers and different hospitals, as the authors discussed, this paper will potentially trigger further analyses for larger scale study as well as the mechanistic study about the relationship between HCV infection and circadian. The specific comments to improve the paper are shown below.

About the background of patients between two groups, it is reported that IL28B genotypes is associated with HCV recurrence after liver transplantation (Charlton et al. Hepatology 53: 317-324, 2011, and other papers). It would be nice to show the IL28B genotypes of donor/recipient of the two groups to neglect the possibility of the potential influence by different IL28B genotypes.

The average duration of operation in the PM group (4.5 h) was shorter than that in the AM group (5.7 h). Showing an evidence or some citations suggesting that such difference will not have a significant impact on the clinical outcome is needed, especially for the present paper suggesting a surprising conclusion.

Fig. 3 shows an interesting result that a SR-BI inhibitor cancelled the difference in HCV recurrent dynamics observed in non-treatment patients. Does it mean that SR-BI is regulated by circadian mechanism and the SR-BI inhibitor treatment reduces this influence on HCV infection? If it does, is there any information or previous papers suggesting that SR-BI is regulated by circadian mechanism? Or if it does not, discussion about the mechanism of the phenotype induced by the SR-BI inhibitor would be helpful.

The authors described “supporting a role for circadian regulation of HCV entry” in line 5 of the right column on page 2, on which more information or citations are needed.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Sep 2018

Peter Balfe, University of Birmingham, UK

Referee : "It would be nice to show the IL28B genotypes of donor/recipient of the two groups to neglect the possibility of the potential influence by different IL28B genotypes."

Response : We thank the reviewer for this excellent suggestion, however, due to ethical issues it was not possible to genotype donor liver IL28B status. Since HCV early replication kinetics is most likely to be defined by the donor liver we decided to not genotype the transplant recipient.

Referee : "The average duration of operation in the PM group (4.5 h) was shorter than that in the AM group (5.7 h). Showing an evidence or some citations suggesting that such difference will not have a significant impact on the clinical outcome is needed, especially for the present paper suggesting a surprising conclusion."

Response : The small cohort size means that a single observation can have a large impact. In this cohort there is one case (patient 1) where the operation took 10h (see on line data file), excluding this atypical data point resulted in mean operation times of 4.5h and 5h, respectively.

Referee : "Fig. 3 shows an interesting result that a SR-BI inhibitor cancelled the difference in HCV recurrent dynamics observed in non-treatment patients. Does it mean that SR-BI is regulated by circadian mechanism and the SR-BI inhibitor treatment reduces this influence on HCV infection? If it does, is there any information or previous papers suggesting that SR-BI is regulated by circadian mechanism? Or if it does not, discussion about the mechanism of the phenotype induced by the SR-BI inhibitor would be helpful."

Response : We previously reported that the SR-BI antagonist ITX5061 inhibited HCV rebound post liver transplant (Rowe et al. 2016) and hence it was not possible to discern any time-of-day effect in the treated arm of this trial.

Referee : "The authors described "supporting a role for circadian regulation of HCV entry" in line 5 of the right column on page 2, on which more information or citations are needed."

Response : There is no additional information or citations showing a role for the circadian regulation of HCV entry factors. We have therefore revised this sentence to read 'suggesting' rather than 'supporting'.

Competing Interests: No competing interests were disclosed.

Referee Report 17 August 2018

doi:[10.21956/wellcomeopenres.16003.r33651](https://doi.org/10.21956/wellcomeopenres.16003.r33651)



Salim I. Khakhoo

Clinical and Experimental Sciences, Faculty of Medicine, Sir Henry Wellcome Laboratories, Southampton General Hospital, University of Southampton, Southampton, UK

The authors have performed a novel study investigating the role of the circadian clock in HCV infection. They have studied 13 patients with chronic HCV infection undergoing liver transplantation either for liver failure or for hepatocellular carcinoma. The study was performed on the back of a trial of an HCV entry inhibitor (ITX5061), which is designed to block reinfection of the liver allograft. They have performed serial HCV viral load measurements in the plasma post transplantation to determine the HCV kinetics during the week after transplantation. They stratify patients into those transplanted in the morning and those in the afternoon. They find that individuals transplanted in the morning were more likely to have had a rebound in viral load. This rebound begins 24-48 hours after the anhepatic phase in most of the individuals and in fig 3A appears to peak at around day 5. The sample size is small but in the untreated individuals is significant. This effect appears lost in the treated group. Overall the study is well conducted and interesting

Comments

1. It is not clear why chose the authors have chosen the specific intervals. Is there a biological rationale, or was it for sample size convenience?
2. It is not clear to me which patients had received ITX5061 (this could be indicated in Figure 2).
3. The number of patients in each group should be indicated in Figure 3.
4. In figure 3 there are treated and untreated individuals. There are 13 patients in Figure 3C and an additional 9 patients in Figure 3D. These do not appear to be included in Table 1. They should be included.
5. How ITX5061 was used is not clear to me. Was it a single dose? Multiple doses? An Infusion? They need to clarify the statement "supporting a role for circadian regulation..." P2 para 1, as presumably it was given during the anhepatic phase for all patients.
6. How does the timing reflect doses of immunosuppression given? Could this also have an effect on the post-transplant kinetic?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Hepatology, immunology viral hepatitis

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 04 Sep 2018

Peter Balfe, University of Birmingham, UK

Referee: "It is not clear why chose the authors have chosen the specific intervals. Is there a biological rationale, or was it for sample size convenience?"

Response : The intervals were selected to maximise sampling from the earliest times post transplant (4 hourly) and to monitor the treated patients throughout the time of ITX5061 administration (see Rowe et al 2016 for details).

Referee list of detailed comments, with our responses interleaved in *italics* :

1. It is not clear to me which patients had received ITX5061 (this could be indicated in Figure 2).
None of the patients in Fig.2 received ITX5061. The treated patients are only shown in Fig.3B, this is now stated in the figure legend.
2. The number of patients in each group should be indicated in Figure 3.
We have edited the figure legend to include these data
3. In figure 3 there are treated and untreated individuals. There are 13 patients in Figure 3C are 13 and an additional 9 patients in Figure 3D. These do not appear to be included in Table 1. They should be included.
Clinical information for the ITX5061 treated patients was previously published (Rowe 2016) and we cited this publication rather than duplicating data in current report.
4. How ITX5061 was used is not clear to me. Was it a single dose? Multiple doses? An Infusion? They need to clarify the statement "supporting a role for circadian regulation..." P2 para 1, as presumably it was given during the anhepatic phase for all patients.
ITX5061 administration details were provided in our earlier report (Rowe 2016) and we cited this publication rather than duplicating data in current report.
5. How does the timing reflect doses of immunosuppression given? Could this also have an effect on the post-transplant kinetic?

All patients arriving at the intensive care unit received standard immunosuppressive therapy of corticosteroids together with tacrolimus and azathioprine (details provided in Rowe 2016). Since our report studied HCV replication in the first week post-transplant when patients received the same immunosuppressive regimen we can exclude this parameter as a confounding influence.

Competing Interests: No competing interests were disclosed.

Referee Report 14 August 2018

doi:[10.21956/wellcomeopenres.16003.r33654](https://doi.org/10.21956/wellcomeopenres.16003.r33654)



William L. Irving 

NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust, University of Nottingham, Nottingham, UK

This paper describes HCV viral replication kinetics in patients in the 7 day period following liver transplantation. To do this, the authors had access to a highly unique set of samples from patients undergoing transplantation in the control arm of a clinical trial of a potential HCV entry inhibitor. The key observation was made that in 4 of 6 patients whose transplant operation occurred between the hours of 6am to 1pm (the AM group), viral load rebounded to pre-transplant levels within the week of follow-up, compared to 0 of 7 patients whose operation took place between the hours of 2pm and 11pm (the PM group). The difference in viral replication kinetics of the 2 groups was statistically significant. For trial patients who received the trial entry inhibitor, there was no difference in replication kinetics. The time of transplant had minimal effect on the initial decline in viral load (clearance of virus from the periphery by the new allograft) in the first 16 hours post-transplantation, but rebound of viral replication, assessed as "the area over the infection curve") was greater in the AM group, although this did not achieve statistical significance, most likely due to the small numbers of patients in each group. In summary, the data are strongly suggestive of an association between HCV allograft infection rate and the time of day of transplantation, and the authors interpret these data to suggest a role for circadian processes to regulate HCV entry into the liver.

The authors acknowledge 2 major limitations to this work – firstly, the small numbers of patients studied, and secondly, the absence of more direct data relating to the circadian status of the donor liver, for which there are obvious ethical constraints. Nevertheless, the observations made using this unusual set of samples are of interest in themselves, and raise further questions. If I have interpreted the data correctly, the implication is that whilst all of the implants appear to take up virus equally well, at least in the first 16 hours, it is only livers implanted in the AM period where the expression of factors underpinning either viral entry or those necessary for rapid viral replication (or both) just happen to be at an optimal level such that viral rebound becomes apparent soon after that initial 16 hour "mopping-up" period. I would be interested to hear the authors' views on two issues related to this:

1. How important might be the time of day of removal of the donor liver? Is it a fair assumption that the circadian status of this liver will become fixed (almost literally frozen) as it is removed from neuronal and hormonal influences at the time of removal? Presumably data on time of removal will be available, although this is not mentioned in the manuscript. Was there also a correlation between viral replication kinetics in the recipient and time of day of removal of the donor organ?
2. A related question, to which the answer most probably will be pure speculation, is how long would it take for the donor liver to become synchronised to the circadian pattern of the recipient? The liver

would be exposed to circulating humoral factors immediately, but there would be an absence of neuronal connections.

A couple of minor points for clarification:

1. The mean duration of the transplant operation was around 5 hours. So would it be true to say, for instance, that there were no operations that began at 11am and continued until 4pm ie straddling the time definitions for AM and PM? Or if that is not true, then into which category would such an operation be classified – AM according to the start time or PM according to the finish time?
2. The authors dismiss any possible confounding by the age of the donor liver – but the median age of the PM livers, at 44 years, was less than the entire range of the AM livers (45-69 years, 1st para of the results). Liver age is a critical factor in both patients with chronic HCV infection (the older age at infection, the more likely disease progression) and the long-term prognosis of liver transplant recipients. Is it not conceivable that this might also be a factor in the PM group being better able to suppress HCV replication, at least in the early post-transplantation phase? Was the donor age in the 4 AM patients who rebounded any different from the donor age of the remaining patients?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 04 Sep 2018

Peter Balfe, University of Birmingham, UK

Referee : "How important might be the time of day of removal of the donor liver? Is it a fair assumption that the circadian status of this liver will become fixed (almost literally frozen) as it is removed from neuronal and hormonal influences at the time of removal? Presumably data on time of removal will be available, although this is not mentioned in the manuscript. Was there also a correlation between viral replication kinetics in the recipient and time of day of removal of the donor organ?"

Response : This is an interesting question but as far as we are aware there are no previous reports

on the circadian status of human liver explants. The cold ischaemic time of the donor livers are provided in the online material, from which time of explant can be calculated. There was no significant difference in the cold ischaemic time between the AM and PM groups and no association with post-transplant HCV replication kinetics.

Referee : "A related question, to which the answer most probably will be pure speculation, is how long would it take for the donor liver to become synchronised to the circadian pattern of the recipient? The liver would be exposed to circulating humoral factors immediately, but there would be an absence of neuronal connections."

Response : A really interesting question to which there is no authoritative answer. PHH explanted from per2-luc transgenic mice can be synchronized in tissue culture and their circadian rhythm lasts around one week (Geunthner et al, PLoS One 2014

<https://doi.org/10.1371/journal.pone.0087573>). Furthermore their circadian rhythm can be re-synchronized by changing the culture media. This suggests that the circadian rhythm of liver explants is highly regulated by environmental factors such as hormones. From this one can speculate that the donor liver will rapidly adapt to the recipients' rhythm post-transplantation. A related question, to which the answer most probably will be pure speculation, is how

Referee : "The mean duration of the transplant operation was around 5 hours. So would it be true to say, for instance, that there were no operations that began at 11am and continued until 4pm ie straddling the time definitions for AM and PM? Or if that is not true, then into which category would such an operation be classified – AM according to the start time or PM according to the finish time?"

Response : The length of all operations are listed in the online data, and with the exception of patient 1 in the PM group (10h), took 4 – 6h to complete. Many AM group patients did not arrive on the ICU until after 14:00. The end of the operation was defined as time of arrival/check in on the post-operative care ward, a significantly less precise parameter than the moment when anhepatic phase began. We therefore selected the start of the anhepatic phase as our time point to stratify the AM or PM groups. A second parameter to consider is the time of liver reperfusion, which occurred within 90 minutes of the anhepatic start time in the majority of cases. Indeed, if one stratifies patients by this criterion, the same groupings are obtained.

Referee : "The authors dismiss any possible confounding by the age of the donor liver – but the median age of the PM livers, at 44 years, was less than the entire range of the AM livers (45-69 years, 1st para of the results). Liver age is a critical factor in both patients with chronic HCV infection (the older age at infection, the more likely disease progression) and the long-term prognosis of liver transplant recipients. Is it not conceivable that this might also be a factor in the PM group being better able to suppress HCV replication, at least in the early post-transplantation phase? Was the donor age in the 4 AM patients who rebounded any different from the donor age of the remaining patients?"

Response : The age of all donors is provided in the online data. The four rebound cases received livers from donors aged 45, 56, 54 and 56 (average 53). One of the donors for the AM group was unusually old (69) and one of the PM donors unusually young (29), if these two cases are excluded then average age for the groups are 53 and 48 years, respectively, supporting our conclusion that donor liver age is unlikely to explain the different viral replication kinetics in these two groups. However, as stated earlier greater patient numbers would strengthen our conclusions.

Competing Interests: No competing interests were disclosed.

